Impact of Acamprosate on Plasma Amyloid-β Precursor Protein in Youth: A Pilot Analysis in Fragile X Syndrome-Associated and Idiopathic Autism Spectrum Disorder Suggests a Pharmacodynamic Protein Marker

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Abstract

Background—Understanding of the pathophysiology of autism spectrum disorder (ASD) remains limited. Brain overgrowth has been hypothesized to be associated with the development of ASD. A derivative of amyloid-β precursor protein (APP), secreted APPα (sAPPα), has neuroproliferative effects and has been shown to be elevated in the plasma of persons with ASD compared to control subjects. Reduction in sAPPα holds promise as a novel molecular target of treatment in ASD. Research into the neurochemistry of ASD has repeatedly implicated excessive glutamatergic and deficient GABAergic neurotransmission in the disorder. With this in mind, acamprosate, a novel modulator of glutamate and GABA function, has been studied in ASD. No data is available on the impact of glutamate or GABA modulation on sAPPα function.

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Methods—Plasma APP derivative levels pre- and post-treatment with acamprosate were determined in two pilot studies involving youth with idiopathic and fragile X syndrome (FXS)-associated ASD. We additionally compared baseline APP derivative levels between youth with FXS-associated or idiopathic ASD.

Results—Acamprosate use was associated with a significant reduction in plasma sAPP(total) and sAPPα levels but no change occurred in Aβ40 or Aβ42 levels in 15 youth with ASD (mean age: 11.1 years). Youth with FXS-associated ASD (n=12) showed increased sAPPα processing compared to age-, gender- and IQ-match youth with idiopathic ASD (n=11).

Conclusions—Plasma APP derivative analysis holds promise as a potential biomarker for use in ASD targeted treatment. Reduction in sAPP (total) and sAPPα may be a novel pharmacodynamic property of acamprosate. Future study is required to address limitations of the current study to determine if baseline APP derivative analysis may predict subgroups of persons with idiopathic or FXS-associated ASD who may respond best to acamprosate or to potentially other modulators of glutamate and/or GABA neurotransmission.

Keywords
amyloid precursor protein; autism spectrum disorder; acamprosate; glutamate; biomarker; GABA

Introduction
Autistic disorder (autism) is a childhood-onset neurodevelopmental disorder characterized by social skills and communication deficits combined with interfering repetitive behavior. Autism is the classic type of pervasive developmental disorder (American_Psychiatric_Association 2000), now termed autism spectrum disorder (ASD) (American_Psychiatric_Association 2013). Despite many recent efforts focused on identifying factors contributing to the development of ASD, more than 75% of cases of ASD remain idiopathic (McGrew et al. 2012).

Among biological factors associated with ASD, macrocephaly is a consistently replicated finding affecting up to 20% of children with autism (Aylward et al. 2002; Aylward et al. 1999; Davidovitch et al. 1996; McCaffrey and Deutsch 2005). Brain magnetic resonance imagery (MRI) studies in ASD have noted abnormal total brain volume enlargement in infants and toddlers (Courchesne et al. 2003; Courchesne et al. 2001; Courchesne and Pierce 2005; Sparks et al. 2002). Furthermore, early brain enlargement marked by increased surface area overgrowth seen in youth with autism may be associated with a disruption in cell adhesion (Hazlett et al. 2011). Among factors contributing to the brain overgrowth theory of ASD, pathophysiology, the potential contribution of dysregulation in amyloid-β precursor protein (APP) metabolism has been proposed (Lahiri et al. 2013; Ray et al. 2011; Sokol et al. 2006; Sokol et al. 2011). APP has been associated with Alzheimer’s disease (AD) where the amyloidogenic pathway of APP processing favors cleavage of APP by β-site APP cleaving enzyme or β-secretase (BACE1) resulting in neurotoxic amyloid-β (Aβ) peptides consisting 40 and 42 amino acids residues (Lahiri et al. 2003). Aβ40 and Aβ42 are the major components of senile plaques associated with brain atrophy in AD. BACE1, which plays a rate-limiting role in the production of potentially toxic Aβ within brain, is an important drug...
target for AD, and indeed, several BACE1 inhibitors are tested in clinical drug trials (Lahiri et al, 2014).

APP is predominantly located at the synapse (Mattson and Furukawa 1998), produced in brain microglia, astrocytes, oligodendrocytes, and neurons (Mullan and Crawford 1993), and released in an activity driven fashion (Jolly-Tornetta et al. 1998). Activation of metabotropic glutamate receptor type 1 and type 5 (mGluR1/5) increases APP secretion in cell culture (Jolly-Tornetta et al. 1998). The highest levels of APP occur early in synaptogenesis (Priller et al. 2006) and peak before 1 month of age in rodents (Lahiri et al. 2002). APP has been implicated in neurite outgrowth (Mattson and Furukawa 1998; Mullan and Crawford 1993) and promotes growth cone development working in opposition to N-methyl-D-aspartate (NMDA) and (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) AMPA glutamate receptors' pruning effects on growth cones (Mattson and Furukawa 1998). As shown in cell culture studies, APP may block and reverse glutamatergic inhibition of dendrite outgrowth (Mattson 1994). APP has been linked to suppression of neuronal cell adhesion (Schubert et al. 1989) and overexpression of APP accelerates migration of neuronal precursor cells into the cortex (Young-Pearse et al. 2007). The non-amyloidgenic APP processing pathway involving cleavage by the α-secretase family of enzymes (such as ADAM 9, 10 and 17) is the predominant APP processing pathway leading to release of non-amyloidgenic secreted APPα (sAPPα) (Mattson 1994; Ray et al. 2011). Several reports have noted neurotrophic effects of sAPPα, including activity in inducing cellular proliferation including the proliferation of neural progenitor cells (Mattson 1997; Stein and Johnson 2003; Turner et al. 2003). Notably, sAPPα also activates microglia (Barger and Harmon 1997). Overall, APP and specifically sAPPα are prime candidates to contribute to synaptic disruption and brain overgrowth in ASD given the proteins' enhancement of neural proliferation. APP modulation and Aβ have been shown to be a target of several drugs, including cholinesterase inhibitors and a partial NMDA receptor antagonist (memantine) (Greig et al. 2005; Lahiri et al. 1994; Alley et al. 2010).

There have been several reports on abnormalities in secreted APP and specifically sAPPα in the blood of youth with autism (Bailey et al. 2008; Ray et al. 2011; Sokol et al. 2006). Higher levels of plasma sAPP total and sAPPα were identified in a small sample of young children with autism and aggressive behavior compared to less impaired youth with autism without aggressive behavior and control subjects (Sokol et al. 2006). In a follow-up report involving 16 youth with autism and 18 control subjects, a similar increase in sAPPα was found in children with severe autism compared to youth with milder cases of autism and neurotypical control subjects (Ray et al. 2011). In the same study, reduced levels of Aβ40 and Aβ42 were detected in the youth with severe autism compared to control subjects. In a study involving 25 youth with autism aged 2-5 years and matched control subjects mean plasma sAPPα was significantly elevated in those with autism; 60% of those with autism had elevations in sAPPα (Bailey et al. 2008). Considering these results together, elevation in plasma sAPP (total) and specifically sAPPα could be a marker of molecular dysregulation contributing to the pathophysiology of autism.

Alterations in brain APP have also been reported in idiopathic ASD. Wegiel and colleagues (2012) have shown abnormal intracellular accumulation and extracellular Aβ deposition in
the brain of persons with ASD (Wegiel et al. 2012). APP expression in brain has been demonstrated to be altered dependent on subject age and brain region in post-mortem ASD specimens (Fatemi et al. 2013).

Monogenetic disorders associated with co-morbid ASD hold promise to provide insight in to the pathophysiology of idiopathic autism. Fragile X syndrome (FXS) is the most common single gene cause of ASD, responsible for approximately 3% of autism cases (Kosinovsky et al. 2005). The prevalence rate of ASD in FXS is estimated between 25% and 50%, depending on the criteria utilized (Clifford et al. 2007; Garcia-Nonell et al. 2008; Hatton et al. 2006; Kaufmann et al. 2004). FXS results from a CGG triplet repeat expansion in the promoter region of the Fragile X Mental Retardation gene (FMR1) on the long arm of the X chromosome. This expansion leads to gene methylation and silencing and subsequent deficiency in Fragile X Mental Retardation Protein (FMRP) production. FMRP is a known repressor of neuronal mRNA translation (Darnell et al. 2011; Lee et al. 2010; Veneri et al. 2004; Zalfa et al. 2003; Zou et al. 2008) and thus is important to synaptic plasticity and regulation of local protein synthesis at the synapse (Bagni and Greenough 2005). Similar to reports in idiopathic ASD, children with FXS also exhibit early brain overgrowth (Hazlett et al. 2012). Additionally, FMRP has been demonstrated to regulate APP mRNA expression (De Rubeis and Bagni 2010; Westmark and Malter 2007). Specifically, FMRP mediates mGluR5-dependent translation of APP mRNA. In normal brain development, activation of mGluR5 neuroreceptors results in suppression of FMRP translational repression of APP, a phenomenon absent in FXS (Westmark and Malter 2007). Baseline APP levels are elevated in Fmr1 knockout (KO) mouse synaptoneurosomes and primary neurons, and APP levels do not increase following mGluR5 stimulation (Westmark and Malter 2007). A preliminary study reported a relative elevation of sAPPα, Aβ40, and Aβ42 in 18 youth with FXS compared to age-matched control subjects (Lahiri 2011). Additionally, Westmark and colleagues (2011) have demonstrated abnormal levels of Aβ42 in the plasma of persons with FXS. Findings from FXS point to enhanced translation of APP compared to potential specific enhancement of the sAPPα synthesis pathway noted in idiopathic ASD. Together, these data from monogenetic and idiopathic ASD converge to support the role of APP dysregulation, specifically excessive levels of sAPPα, in the pathophysiology of ASD. Given this, it is important to explore elevated sAPPα expression as a target of treatment in ASD. Conceptually, the three proteins APP, FMRP, and mGluR5 are proposed to serve as molecular links for ASD, AD, and FXS, and consequently any disruption in interaction of this “trinity” could lead to the disease phenotype (Lahiri et al. 2013). It may be that analyses of APP metabolites and APP processing enzymes hold promise as a target of treatment, pharmacodynamic marker of target engagement, and as a potential means to subgroup populations matched to potential treatments of interest.

Acamprosate is approved by the United States Food and Drug Administration (FDA) for the maintenance of abstinence from alcohol use in adults. It is a novel agent with multiple potential mechanisms of action demonstrated in animal studies. Potential pharmacodynamic properties include attenuation of N-methyl-D-aspartate (NMDA) (Mayer et al. 2002; Naassila et al. 1998) and metabotropic type 5 glutamate receptor (mGluR5) (Blednov and Adron Harris 2008; Gupta et al. 2008; Harris et al. 2002; Kotlinska and Bochenski 2008)
neurotransmission and agonist effects at gamma-aminobutyric acid type A (GABA(A))
neuroreceptors (Mann et al. 2008; Pierrefiche et al. 2004). The exact mechanisms of action
of acamprosate remain unclear, in part, given results of an electrophysiology receptor
binding study that noted no direct activity of acamprosate at glutamate or GABA receptors
using clinically relevant drug concentrations (Reilly et al. 2008). Given excessive
glutamatergic and deficient GABAergic neurotransmission have been noted in studies of
humans with idiopathic ASD noted in idiopathic ASD (McDougle et al. 2005), the
pharmacodynamic properties of acamprosate may be well matched with the pathophysiology
of ASD. Also, acamprosate use in FXS-associated ASD is supported by extensive bodies of
literature noting excessive mGluR5 (Bear 2005; Bear et al. 2004) and deficient GABA(A)
(Bear 2005; Bear et al. 2004) neurotransmission in this disorder.

In a preliminary report, potential improvement in communication was described with open-
label acamprosate treatment in 3 adults with FXS and ASD (mean duration: 21.3 weeks;
mean dose= 1,221 mg/day) (Erickson et al. 2010). Subsequently, a 10-week prospective
open-label trial of acamprosate (mean dose= 1,054 ± 422 mg/day) in 12 youth (mean age:
11.9 years) with FXS and comorbid ASD was completed (Erickson et al. 2013b). In this
study, acamprosate was associated with positive clinical response in 9 subjects (75%) with
specific improvement noted in social impairment and inattention/hyperactivity (Erickson et
al. 2013b).

In an open-label naturalistic study of acamprosate (mean duration: 20 weeks; mean dose=
1,110 mg/day) in six youth with idiopathic autism (mean age: 9.5 years), the drug was
associated with positive response in five subjects (83%) with improvement primarily noted
in social relatedness (Erickson et al. 2011). More recently, results from a single-blind,
placebo lead-in pilot study of acamprosate in 12 youth with idiopathic ASD were reported.
In this report, 6 of 9 (67%) of youth with ASD who received acamprosate showed clinical
response characterized by improvement in social relatedness and inattention/hyperactivity
(Erickson et al. 2013a).

Given several factors, including the need to better understand the pharmacodynamic effects
of acamprosate in ASD, the potential importance of APP dysregulation in the
pathophysiology of ASD, and the need for quantitative biomarkers to predict and assess
treatment response in clinical trials of ASD, we now report on the impact of acamprosate
use on APP and its metabolites in youth with idiopathic and FXS-associated ASD. We
hypothesized that acamprosate use would be associated with reductions in APP and its
metabolites, specifically sAPPα, and that these changes would correlate with positive
response to acamprosate.

Materials and Methods

Levels of total sAPP (total), sAPPα, Aβ40, and Aβ42 were analyzed from plasma specimens
of subjects participating in a pilot 10-week open-label study of acamprosate in 12 youth
aged 5-17 years with full mutation FXS and comorbid ASD (Erickson et al. 2013b) or in a
pilot study in 12 youth aged 5-17 years with idiopathic autism who, following a 2-week
placebo lead-in, received 10 weeks of single-blind acamprosate treatment (Erickson et al.
For all subjects, two plasma samples were compared, baseline sampling prior to study drug treatment and sampling at final study visit. All subjects remained on stable concomitant psychotropic drug dosing throughout the pilot trials with the exception of use of modulators of glutamate or GABA(A) neurotransmission which were prohibited.

Blood samples were collected in tubes containing EDTA (K$_2$EDTA from Becton Dickinson, Franklin Lakes, NJ, USA, product #367863). Test plasma samples were prepared soon after collection. Briefly, plasma was isolated from freshly drawn blood by centrifuging at 1000×g for 12 minutes. The isolated plasma samples were further centrifuged at 10,000×g for 10 minutes for complete removal of platelets. Prepared plasma samples were aliquoted in several microfuge tubes and stored at -80°C to avoid repeat freeze-thaw. The test samples were thawed on ice just before use. If necessary, the plasma samples were diluted appropriately with the EIA buffer, and the appropriate assay was performed in duplicate measurements for the test samples and standards. Test samples in neutral pH range were used, and steps were taken to avoid the contamination from organic solvents. Regarding the standard to quantify the sAPP$\alpha$ levels, a series of sAPP$\alpha$ standards were prepared in EIA buffer by serial dilutions from 0.78 ng/mL to 50 ng/mL. Most of the procedure have recently been reported by us$^{15}$.

The ELISA plates were pre-coated with highly specific anti-human affinity purified sAPP$\alpha$ (2B3) mouse IgG-monoclonal antibody (IBL America). First, the wells for the reagent blank were determined, and 100 μL each of “EIA buffer” buffer was placed into the blank wells. Likewise, different wells were assigned for the test samples and diluted standards. Next, 100 μL each of test samples and dilutions of standards in EIA buffer were added into the appropriate wells. The test sample included the plasma sample from each subject, which may vary from 5-25 μL. The pre-coated plate was incubated overnight at 4°C. After several washes, 100 μL of labeled antibody solution was added into the wells of test samples, diluted standard and of test sample blank. HRP-conjugated and labeled anti- Human APP (R101A4) mouse IgG from IBL was used. Each plate was incubated for 30 minutes at 4°C and then washed several times. ELISA signals were developed by adding TMB buffer followed by the addition of Stop buffer 1(N) H$_2$SO$_4$. Using a plate reader (BioRad) measurements were conducted at 450 nm against a reagent blank. The measurement was done within 30 minutes addition of the Stop solution. Before performing the ELISA with all plasma samples, different volumes of a “pool” plasma sample were analyzed to establish the linearity of the assay.

To determine sAPP levels, we thawed the test samples at a low temperature and mixed them completely. Regarding the standard to quantify levels of sAPP, we prepared a series of sAPP standards in EIA buffer by serial dilutions, from 0.39 ng/mL to 25 ng/mL. The ELISA plate was pre-coated with anti-human APP (R12A1) mouse IgG (IBL). ELISA of plasma samples was carried out as per the manufacturer's protocol and similar to the method described above. This ELISA kit uses HRP-labeled anti- Human APP (R101A4) mouse IgG as the detection antibody. Levels of A$\beta$ peptides were assayed in platelet free plasma samples by an ultra sensitive and specific ELISA (Wako Chemical Industries, Japan). Plasma samples were diluted 2-10 times to avoid nonspecific signals. The ELISAs use highly specific capture antibodies BA27 and BC05 to detect A$\beta$ (1-40) and A$\beta$ (1-42), respectively. The
overall assay procedures were performed as per the guidelines of the manufacturer. ELISA of the plasma samples were performed in a ‘blinded’ manner.

The primary outcomes of this report are comparison of plasma levels of sAPP-total, sAPPα, Aβ40, Aβ42, as well as the ratios of sAPPα/sAPP and Aβ42/Aβ40 pre- and post-acamprosate treatment. All data were coded into IBM SPSS Statistics 21 or SAS 9.4 for analysis. The differences between pre- and post- assays were compared by paired t-tests in the pool sample (FXS and idiopathic ASD), by bootstrap resampling of the mean difference in the individual treatment groups and 95% confidence intervals and Hedge's g calculated. An exploratory Kendall's tau correlation analysis was conducted to assess for any relationship between change in primary outcomes above and change in behavioral outcome measures that showed improvement during our pilot clinical trials. This included analysis of the Social Responsiveness Scale (SRS) (Constantino et al. 2003) total score, Aberrant Behavior Checklist Social Withdrawal subscale (ABC-SW) (Aman et al. 1985), ADHD Rating Scale 4th Edition (ADHD-RS) (DuPaul et al. 1998), and Clinical Global Impression Improvement (CGI-I) scale (Guy 1976).

Finally, we conducted an analysis of baselineAPP plasma derivatives in youth with FXS and ASD versus age-, gender- and IQ-matched youth with idiopathic ASD. This sampling involved all subjects enrolled in the FXS pilot clinical trial of acamprosate (pre-treatment analysis) and a group of matched subjects with idiopathic ASD not receiving acamprosate or other glutamate or GABA(A) modulators. Samples were assayed for total soluble APP (sAPP-total), soluble APPα (sAPPα), Aβ42, and Aβ40. The ratios of sAPPα/sAPP and Aβ42/Aβ40 were calculated. Bootstrap estimation of the differences between mean values of idiopathic ASD samples vs. FXS samples was done, instead, with 95% and 90% confidence intervals calculated. Those differences for which the confidence interval did not cross zero were counted as “significant” at p < 0.05 or p < 0.10, as appropriate. In addition, Hedge's g standardized effect sizes were calculated for each marker or ratio.

Results

Fifteen subjects (mean age: 11.1 years, range 6-15 years; mean IQ= 55, range 47-83) had available pre- and post-acamprosate plasma sAPP derivative levels (see Table 1). This sample included 9 youth with FXS-associated ASD (mean age: 10.9 years) and 6 youth with idiopathic ASD (mean age: 11.4 years). Thirteen subjects received concomitant psychotropic drugs (mean number of drugs= 1.9) at stable doses during the trial period (see Table 2). The mean final acamprosate dose for subjects in this analysis was 1,061 mg/day. Acamprosate treatment significantly (p < 0.05) reduced levels of overall sAPP-total and sAPPα in both subjects with FXS-associated or idiopathic ASD in the pooled sample and divided by treatment group (FXS versus idiopathic ASD; Tables 3-4; Figure 1). Acamprosate treatment also increased the sAPPα/sAPP ratio in both groups. However, several of the markers measured appeared to have a single large outlier. A crude validation was done by leaving out the most extreme individual difference of each marker or ratio and repeating the analysis. When this was done, the reductions in sAPP and sAPPα were maintained. The increase in the mean sAPPα/sAPP ratio following acamprosate treatment lost significance in the idiopathic ASD group. On the other hand, this produced a significant
result for reduced Aβ42 levels in the FXS-associated ASD group. These results suggest that acamprosate treatment reduces overall sAPP levels in both FXS-associated ASD and idiopathic ASD, and that this may be accompanied by a somewhat greater redirection toward the α-secretase processing pathway in FXS-associated than in idiopathic ASD.

Our exploratory Kendall’s tau correlation analysis noted no correlation between change in plasma sAPP-total, sAPPα, Aβ40, or Aβ42 and change on the SRS, ABC-SW, ADHD-RS, or CGI-I in the pooled patient sample (n=15). In the FXS-associated (n=9) subgroup, change in ABC-SW scores correlated with change in sAPP-total (p=0.009) and sAPPα (p=0.04). No significant correlations were noted in the idiopathic ASD (n=6) subgroup.

In our comparison of baseline plasma APP derivatives between youth with FXS-associated ASD (n=12; mean age: 11.9 years) versus those with idiopathic ASD (n=11; mean age: 11.8 years; see Tables 5-6), all individual markers analyzed had higher levels in the FXS-associated ASD group (Table 7; Figure 2). This was also true for the sAPPα/sAPP ratio. Of the differences, only sAPPα/sAPP was significant at p < 0.05, suggesting that youth with FXS-associated ASD have greater levels of pro-neurotropic sAPPα species than those with idiopathic ASD. Levels of Aβ42 were higher in FXS-associated ASD at p < 0.10. Aβ40 was also higher in FXS-associated versus idiopathic ASD, although the difference was not significant. Overall, there was a tendency toward greater APP-related protein and peptide products in plasma of youth with FXS-associated ASD. Crude validation was done by repeating the analysis while excluding the most extreme value for each marker or ratio within the FXS and idiopathic autism samples. When this was done, the difference between FXS-associated and idiopathic ASD for Aβ42 levels was accompanied by significance of p < 0.05 instead of p < 0.10. On the other hand, the difference for sAPPα/sAPP lost significance of p < 0.05, but remained within p < 0.10. Thus, our conclusions should be taken with some caution, but permitting the possibility of better resolution in a larger study.

**Discussion**

This is the first report on potential change in plasma proteins sAPP-total and sAPPα, and peptides Aβ40, and Aβ42 following drug treatment in youth with ASD. Due to inaccessibility of brain tissue samples from living subjects, we have argued recently that the plasma levels of these “neuronal” proteins would reflect the change occurring in the CNS (Ray et al. 2011; Sokol et al. 2011). Given the implication of APP dysregulation in the pathophysiology of ASD, it will be important to consider in particular elevations of sAPPα as a measurable target of treatment of the disorder. Baseline profiling of plasma APP derivatives may also hold promise in molecular endophenotyping as a means to potentially predict targeted treatment response in future ASD clinical trials.

We additionally reported on novel comparison of sAPP derivative plasma levels in youth with FXS-associated or idiopathic ASD matched on age, IQ, and gender. This comparison controlled for the diagnosis of ASD to see if differences in the APP processing pathway may exist due to FXS. Our initial data points towards increased pro-neurotrophic sAPPα in FXS-associated versus idiopathic ASD. Without a neurotypical control group in this report, further interpretation is limited. Despite this, our findings may represent, based on extension
from previously reported data (Bailey et al. 2008; Ray et al. 2011; Sokol et al. 2006; Sokol et al. 2011), a continuum of excessive non-amyloidogenic APP processing where both FXS-associated and idiopathic ASD show a non-amyloidogenic processing bias compared to controls, with FXS being potentially associated with the greatest non-amyloidogenic processing bias.

The reduction in total sAPP and sAPP\(\alpha\) levels independently with acamprosate treatment takes an important step in understanding the potential molecular mechanisms of this drug in persons with ASD. This is of particular importance given the implication of specifically excessive sAPP\(\alpha\) production in persons with ASD. This work begins to demonstrate engagement of a novel drug treatment with a putative molecular marker of pathophysiology in a disorder devoid of validated biomarkers for use in clinical trials. The specific mechanisms by which acamprosate may lead to reduction in total sAPP and sAPP\(\alpha\) remain to be understood. Our hypothesis is that potentially direct or indirect attenuation of glutamate receptor, specifically mGluR5, activity may lead to reengagement of a transcriptional break on APP production given preclinical reports describing control of APP translation in FXS animal models.

The results of this work must be taken in the context of the multiple limitations of the study design. Our primary limitation is the sample size with pre- and post-acamprosate sampling from only 15 study subjects. Given this, our ability to detect more subtle drug effects and/or begin to understand correlations between change in sAPPtotal, sAPP\(\alpha\), Aβ40, or Aβ42 and change in behavioral outcome measures is very limited. Our lack of a positive correlation finding of APP derivative change and outcome measure findings may be due to this weakness. Additionally, while the APP analysis was conducted blinded to study assignment, the open-label nature of the drug trials introduced bias into the behavioral outcome assessments thus further confounding our correlation analysis.

The use of concomitant psychotropic drugs at stable doses may potentially alter baseline APP derivative levels thus obscuring the ability to detect change with acamprosate treatment. For example, rivastigmine has been demonstrated to lower Aβ and increase sAPP\(\alpha\) levels (Bailey et al. 2011). Despite these limitations, a clear signal of significant reduction in plasma sAPPtotal and sAPP\(\alpha\) levels following acamprosate treatment was noted. Inexact matching in baseline medication use may have also impacted our APP derivative comparisons between youth with FXS-associated and idiopathic ASD. Despite this, our overall patterns of concomitant medication use were quite similar among groups (see Table 5). Future placebo-controlled study potentially stratifying based on concomitant medication use patterns or study prohibiting concomitant medications will be necessary to fully understand this issue.

Our use of plasma measurement essentially as a proxy for what may occur within the brain is a potential weakness of our analysis. We recognize the fact that it would be difficult to have a direct correlation between plasma and brain levels of APP metabolites due to, among other factors, differences in the PD and PK profile of the drug tested in different tissues. There are very few reports of parallel measurements of proteins and other molecules in both plasma and brain tissue samples (Tajima et al. 2013). There is an interesting report of an in
vivo evaluation of avagacestat (a γ-secretase inhibitor) in dogs, which showed a plasma half-life that supports daily oral dosing, good brain penetration, and a correlation between reductions of Aβ40 levels in the brain and cerebrospinal fluid (CSF)(Albright et al. 2013).

Future directions based upon our findings include use of plasma APP derivative analysis both in larger samples of persons with FXS-associated and idiopathic ASD receiving acamprosate and following use of other potential novel targeted treatments. Currently, we are analyzing plasma levels of sAPPtotal, sAPPα, Aβ40, and Aβ42 pre- and post-treatment in placebo-controlled trials of acamprosate in new cohorts of youth with idiopathic ASD (NCT01813318) or FXS (NCT01911455). It will also be important to consider future pre-clinical methods to parse out what specific drug mechanisms may reduce plasma sAPPtotal and sAPPα. Given the uncertainty surrounding the mechanisms of acamprosate and the likely multiple mechanisms of action of this drug, an improved mechanistic understanding of the pharmacodynamic property(s) necessary to modulate APP derivatives in these populations will be essential to future targeted treatment developments.

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Highlights

- This is the first report on use of plasma amyloid-β precursor protein (APP) analysis pre- and post-targeted treatment in fragile X-associated and idiopathic autism spectrum disorder.
- Acamprosate use was associated with uniform reduction in plasma sAPP (total) and sAPPα levels.
- Targeting elevated plasma sAPP (total) and sAPPα holds promise as a molecular target of treatment in fragile X-associated and idiopathic autism spectrum disorder.
Pre- and Post-Acamprosate in Idiopathic ASD Subject

Fig. 1. Individual Subject Pre- and Post-Acamprosate Change

Effects of acamprosate treatment of idiopathic autistic subjects on selected plasma APP processing products. Six idiopathic autism subjects were treated with acamprosate as described in the text. Plasma samples were taken on day 0 (W0) and after 12 weeks (W12) and assayed for sAPPα, sAPP, Aβ42, and Aβ40. The W0 result for each individual subject was subtracted from its corresponding W12 result, and mean results subject to bootstrap analysis against the null hypothesis that W12 - W0 = 0. Results are presented as “fan plots”, which explicitly link individual W12 to corresponding W0 results. Gray lines show individual subjects. Orange solid lines show mean sample change. Orange dashed lines show mean sample change excluding most extreme result. Blue lines show “null” zero. Figure includes results for A) sAPP, B) sAPPα, C) Aβ42, D) Aβ40, and E) Aβ42/Aβ40. Samples significant at p < 0.05 are shown with “**”, those significant at p < 0.10 with “†”. If validation suggested a potential different level of significance, it is shown in square brackets “[[]]”.

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Fig. 2. Analysis of APP Derivatives in FXS-Associated Versus Idiopathic ASD
Comparison of selected plasma APP processing products in idiopathic autism and fragile X. Plasma samples were collected from 11 idiopathic autism and 12 fragile X subjects and assayed for sAPPα, sAPP, Aβ42, and Aβ40, as described in the text. Difference of means between autism and fragile X were analyzed by bootstrap. Relative mean values (idiopathic autism = 1), with 95% confidence interval error bars are shown. Samples significant at p < 0.05 are shown with “*”, those significant at p < 0.10 with “†”. If validation suggested a potential different level of significance, it is shown in square brackets “[]”.
### Table 1
Characterization of Subjects for Pre- and Post-Acamprosate Treatment Analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Male Subjects</th>
<th>Number of Female Subjects</th>
<th>Mean IQ</th>
<th>Mean Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic ASD</td>
<td>5</td>
<td>1</td>
<td>72</td>
<td>11.4</td>
</tr>
<tr>
<td>FXS plus ASD</td>
<td>7</td>
<td>2</td>
<td>45</td>
<td>10.9</td>
</tr>
</tbody>
</table>
Table 2
Pre- and Post-Acamprosate Treatment Group Concomitant Psychotropic Medication Use

<table>
<thead>
<tr>
<th>Drug</th>
<th>Number of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risperidone</td>
<td>6</td>
</tr>
<tr>
<td>Clonidine</td>
<td>6</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>3</td>
</tr>
<tr>
<td>Methylphenidate ER</td>
<td>3</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>2</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>2</td>
</tr>
<tr>
<td>Guanfacine</td>
<td>2</td>
</tr>
<tr>
<td>Vyvanse</td>
<td>1</td>
</tr>
<tr>
<td>Trazodone</td>
<td>1</td>
</tr>
<tr>
<td>Valproic Acid</td>
<td>1</td>
</tr>
<tr>
<td>Dextromine</td>
<td>1</td>
</tr>
<tr>
<td>Oxcarbazepine</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3
Pre- and Post-Acamprosate Amyloid Precursor Protein Derivative Levels (pooled sample)

<table>
<thead>
<tr>
<th>Plasma Levels</th>
<th>APP Total (ng/mL)</th>
<th>sAPPα (ng/mL)</th>
<th>Aβ40 (pg/mL)</th>
<th>Aβ42 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (±SEM)</td>
<td>32.60 ± 9.90</td>
<td>8.35 ± 2.05</td>
<td>144.67 ± 15.04</td>
<td>35.21 ± 3.49</td>
</tr>
<tr>
<td>Follow-up (±SEM)</td>
<td>21.45 ± 8.34</td>
<td>5.49 ± 1.87</td>
<td>138.70 ± 11.13</td>
<td>32.22 ± 2.99</td>
</tr>
<tr>
<td>P value (Paired T Test)</td>
<td>0.01*</td>
<td>0.003*</td>
<td>0.64</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Difference is significant at p<0.05
### Table 4
Acamprosate Treatment Effects on Plasma APP Processing Products in Idiopathic ASD vs. Fragile X Syndrome Associated ASD

<table>
<thead>
<tr>
<th>Marker</th>
<th>Idiopathic ASD</th>
<th>Fragile X Syndrome</th>
<th>Difference</th>
<th>Hedge’s $g$</th>
<th>Difference</th>
<th>Hedge’s $g$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sAPP</td>
<td>$-13.05 \pm 7.65/18.34^a$</td>
<td>$0.61 \pm 0.66/0.23$</td>
<td>$-8.28 \pm 5.12/15.24^a$</td>
<td>$0.55 \pm 0.20/0.20$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sAPPα</td>
<td>$-2.94 \pm 1.81/4.43^a$</td>
<td>$0.59 \pm 0.64/0.22$</td>
<td>$-1.78 \pm 0.82/2.28^a$</td>
<td>$0.75 \pm 0.25/0.31$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ42</td>
<td>$0.52 \pm 0.95/2.40$</td>
<td>$0.20 \pm 1.30/1.19$</td>
<td>$-1.05 \pm 1.59/0.87$</td>
<td>$0.51 \pm 1.57/0.80$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ40</td>
<td>$0.46 \pm 4.64/8.03$</td>
<td>$0.05 \pm 0.67/1.53$</td>
<td>$-1.28 \pm 7.83/8.34$</td>
<td>$0.09 \pm 0.67/0.74$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sAPPα/sAPP</td>
<td>$0.03 \pm 0.19/0.02^b$</td>
<td>$0.41 \pm 0.59/0.88$</td>
<td>$0.07 \pm 0.08/0.05^a$</td>
<td>$0.66 \pm 0.53/0.44$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ42/Aβ40</td>
<td>$-0.02 \pm 0.20/0.03$</td>
<td>$0.21 \pm 0.71/1.11$</td>
<td>$-0.03 \pm 0.04/0.06$</td>
<td>$0.37 \pm 0.57/0.71$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All data reported as result ± upper CI/lower CI. Units for sAPP and sAPPα are ng/ml. Units for Aβ42 and Aβ40 are pM.

- $^a$ Difference is significant at $p < 0.05$.
- $^b$ Difference is significant at $p < 0.05$, but “leave out extreme” validation removed this significance.
### Table 5
Characterization of Subjects for Baseline APP Derivative Analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Subjects</th>
<th>Number of Females</th>
<th>Mean IQ</th>
<th>Mean Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic ASD</td>
<td>11</td>
<td>2</td>
<td>50</td>
<td>11.8</td>
</tr>
<tr>
<td>FXS plus ASD</td>
<td>12</td>
<td>2</td>
<td>45</td>
<td>11.9</td>
</tr>
</tbody>
</table>
Table 6
Baseline Fragile X Syndrome versus Idiopathic Autism Concomitant Psychotropic Medication Use

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fragile X Syndrome (N)</th>
<th>Idiopathic Autism (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risperidone</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Methylphenidate ER</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sertraline</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Clonidine</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Dextroamphetamine</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Guanfacine</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Lisdexamethasone</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Oxcarbazepine</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mixed Amphetamine</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
### Table 7
Levels of Plasma Aβ42 and Aβ40 in Idiopathic ASD vs. Fragile X Syndrome Associated ASD Subjects

<table>
<thead>
<tr>
<th>Marker or Ratio</th>
<th>Level (relative to idiopathic autism)</th>
<th>Hedge’s g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Idiopathic Autism</td>
<td>Fragile X</td>
</tr>
<tr>
<td>sAPP</td>
<td>1.00 ± 1.05/0.53</td>
<td>1.12 ± 1.24/0.71</td>
</tr>
<tr>
<td>sAPPα</td>
<td>1.00 ± 1.00/0.43</td>
<td>1.09 ± 0.80/0.39</td>
</tr>
<tr>
<td>Aβ42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00 ± 0.19/0.14</td>
<td>1.30 ± 0.25/0.29</td>
</tr>
<tr>
<td>Aβ40</td>
<td>1.00 ± 0.33/0.23</td>
<td>1.22 ± 0.28/0.26</td>
</tr>
<tr>
<td>sAPPα/sAPP&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>1.00 ± 0.20/0.16</td>
<td>1.79 ± 0.93/0.65</td>
</tr>
<tr>
<td>Aβ42/Aβ40</td>
<td>1.00 ± 0.12/0.18</td>
<td>0.98 ± 0.10/0.10</td>
</tr>
</tbody>
</table>

All data reported as result ± upper CI/lower CI

<sup>a</sup> Fragile X and idiopathic samples differed at p < 0.05.

<sup>b</sup> Fragile X and idiopathic samples for Aβ42 differed at p < 0.10.

<sup>c</sup> Hedge’s g greater than 0 at ± 95% confidence interval.